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Review

Structural insights into the SNARE mechanism

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Abstract

Eukaryotic cells distribute materials among intracellular organelles and secrete into the extracellular space through cargo-loaded vesicles. A concluding step during vesicular transport is the fusion of a transport vesicle with a target membrane. SNARE proteins are essential for all vesicular fusion steps, thus they possibly comprise a conserved membrane fusion machinery. According to the ''zipper'' model, they assemble into stable membrane-bridging complexes that gradually bring membranes in juxtaposition. Hence, complex formation may provide the necessary energy for overcoming the repulsive forces between two membranes. During the last years, detailed structural and functional studies have extended the evidence that SNAREs are mostly in accord with the zipper model. Nevertheless, it remains unclear whether SNARE assembly between membranes directly leads to the merger of lipid bilayers.

Keywords: SNARE protein; Membrane fusion; Coiled coil

1. Introduction

In the last decade, studies on vesicular transport have converged on SNARE proteins as being essential for membrane fusion. SNAREs are a family of small membrane proteins that can form stable hetero-oligomeric complexes [1,2]. Their complex-forming regions are located adjacent to their respective C-terminal membrane anchors and consist of homologous coiled-coil regions of approximately 60 residues, termed SNARE motifs [3]. It is believed that SNAREs anchored in the membrane of a transport vesicle pair with SNAREs anchored in the target membrane. The different intracellular transport steps are thought to be mediated by different members of the SNARE family, suggesting that they act via a conserved mechanism (for recent reviews see Refs. [4–10]).

Complex formation between membranes is thought to originate at the N-terminal ends of the SNARE motifs. Their subsequent zippering into stable membrane-bridging (''trans''-) complexes would bring the membranes gradually into close apposition, eventually leading to membrane merger [11,12]. Today, this intuitive ''zipper'' model is widely accepted, but it should be born in mind that the supporting evidence are largely circumstantial. SNAREs reconstituted into liposomes can catalyze slow membrane fusion [13], but other proteins, which in vivo most probably do not work as fusion factors, have been shown to mediate liposome fusion as well [9]. In addition, it remains inconclusive whether in vivo additional protein(s) may catalyze the final step of membrane fusion after the formation of *trans*-SNARE complexes. Unfortunately, so far the utilized liposome fusion assay does not monitor SNAREs directly [13]. In fact, to establish that SNAREs are indeed fusion factors, it seems elementary to structurally characterize the proteins in membranes in greater detail. Over the last years, the increasing structural knowledge of SNARE proteins has enabled for a continuous refinement of the biophysical approaches to follow the dynamics of SNARE assembly. Only a thorough structural, thermodynamic, and kinetic description of SNARE assembly will eventually lead to a more complete understanding of the SNARE mechanism in vivo.

This review primarily focuses on the recent data obtained from studies on the soluble domains of SNAREs and on SNAREs incorporated in membranes. In addition, I shall critically relate the structural and biophysical properties of SNAREs to their proposed activity during membrane fusion.

Abbreviations: NEM, *N*-ethylmaleimide; NSF, NEM-sensitive factor; SNAP, soluble NSF attachment protein; SNARE, SNAP receptor; SNAREs, SNARE proteins; TM, transmembrane; EPR, electron paramagnetic resonance; FRET, fluorescence resonance energy transfer; NMR, nuclear magnetic resonance

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2. SNARE protein structure

In the last years, SNARE proteins have been studied with a variety of structural approaches. To date, the best characterized SNARE proteins are the ones involved in synaptic exocytosis. The synaptic SNARE complex consists of the synaptic vesicle protein Synaptobrevin 2 (also referred to as VAMP 2) and the plasma membrane proteins Syntaxin 1a and SNAP-25. Syntaxin 1a and Synaptobrevin 2 each contain a single SNARE motif directly adjacent to their C-terminal transmembrane (TM) domain. In addition to the SNARE motif, Syntaxin 1a contains an N-terminal domain that spans about two thirds of its sequence. SNAP-25 is composed of two SNARE motifs connected by a linker. It does not possess a TM domain, but is attached to the membrane by palmitoyl modifications in the linker region. Because Synaptobrevin 2 is confined to the vesicular membrane, it has been named ''v-SNARE''; since Syntaxin 1a and SNAP-25 are preferentially located in the plasma membrane (target membrane), they are called ''t-SNAREs''. Their distribution in opposing membranes led to the idea that Syntaxin and SNAP-25 together may form the acceptor site for Synaptobrevin [1,2,14].

2.1. Major conformational changes occur upon SNARE assembly

In solution, the individual SNARE motifs of the synaptic SNARE complex are largely unstructured. Upon assembly, major structural rearrangements take place towards a stable complex with significant α-helical content [15–18]. The transition from unstructured monomers to stable complexes is likely to be a common feature of all SNARE proteins because similar changes have been observed for other SNARE sets [19–22]. In vivo, the structure of SNARE proteins is probably influenced by their membrane environment and their interaction with other proteins. However, the unstructured conformation might be a prerequisite for assembly. Therefore, it is likely that SNAREs occur, at least transiently, in an unstructured conformation in the cellular environment.

Apart from SNARE proteins, several other ''natively'' unstructured proteins have been reported [23]. It is thought that the lack of intrinsic structure can confer functional advantages on a protein by allowing more adaptable binding. Folding induced upon binding is accompanied by an entropic penalty, but is counterbalanced by a large enthalpy of binding. It is plausible that coupling of binding and folding during SNARE complex formation, probably after successful nucleation, allows for a directed and progressive assembly process. The accompanying structural changes may slow down SNARE zippering and render it susceptible to control by other proteins.

The energy released during SNARE assembly [15] may be used to overcome the energy barrier for membrane apposition and fusion. Because of intriguing mechanistic parallels, SNARE-mediated membrane fusion has often been compared to the fusion of enveloped viruses with a host cell [24]. Yet, there is neither sequence nor true structural similarity between the involved machineries. In addition, the two fusion machineries appear to provide free-energy by a somewhat different mechanism. Viral fusion proteins are thought to release energy through a change from a metastable into the thermodynamically most stable conformation [24,25]. Conversely, SNARE proteins appear to assemble from mostly unstructured monomers into a kinetically stabilized, quasi irreversible protein–protein interaction [26], which—to be recycled—has to be disassembled by the ATPase NEM-sensitive factor (NSF) and its SNAP-cofactor [1]. It is hence tempting to speculate that the disassembly machinery served as a ''pre-adaptation'' (exaptation) for the development of an ''irreversible'' SNARE interaction.

2.2. Structure of the assembled SNARE complex

Limited proteolysis experiments demonstrated that the synaptic complex is composed of two independent domains, the N-terminal domain of Syntaxin 1a and the SNARE core complex [27,28]. The N-terminal domain of Syntaxin 1a consists of an autonomously folded three-helix bundle, called Habc-domain [29,30]. The core complex is composed of a tightly packed parallel four-helix bundle, with Syntaxin 1a and Synaptobrevin 2 each contributing one helix and SNAP-25 two helices [31]. The SNAP-25-helices are connected by a flexible linker that extends over the entire length of the bundle [18] (Fig. 1A). In the interior, the helices form 16 layers (numbered from −7 to +8) of interacting amino acid side chains that are mostly hydrophobic. Sequence comparison demonstrated that the layer residues are highly conserved across the SNARE family [32,33]. The overall sequence identities between distant family members, however, are rather limited. An unusual hydrophilic layer exists in the middle of the bundle (''0-layer'') consisting of three glutamine residues (Q), one contributed by Syntaxin 1a and two by SNAP-25, and one arginine residue (R), contributed by Synaptobrevin 2. The arrangement of side chains in several other layers is as well highly asymmetric, indicating that each of the four helices of the bundle is unique and belongs to a different SNARE subfamily. Because of the almost 100% conservation of the remarkable ''0-layer'', we have reclassified SNAREs into Q- and R-SNAREs [33]. The SNARE helices of Syntaxin and of the N-terminal and C-terminal halves of SNAP-25 are named Qa-, Qb-, and Qc-SNARE, respectively [34,35], the Synaptobrevin helix is called R-SNARE.

Their high homology suggests that all other SNARE complexes may have a similar four-helix bundle structure as the synaptic complex. This view was strengthened when the core structure of the only distantly related endosomal SNARE complex was shown to be remarkably similar to that of the synaptic complex [36]. Interestingly, the endo-

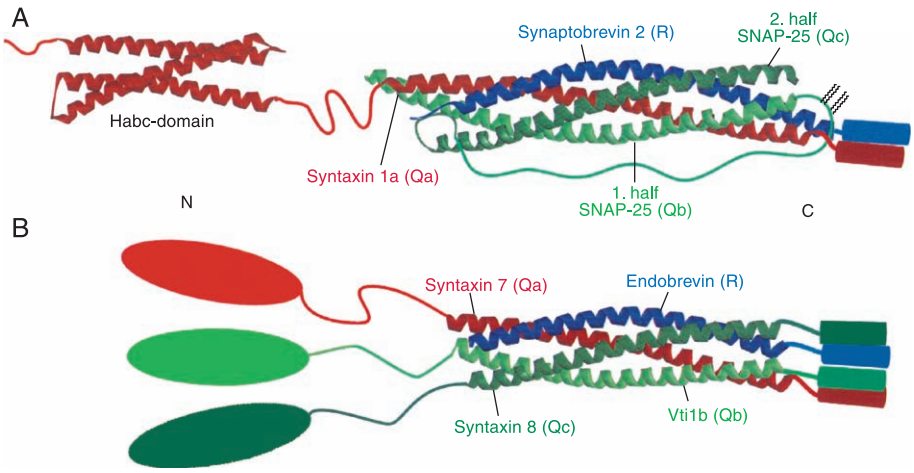

Fig. 1. Model of the synaptic and the endosomal SNARE complex. (A) The core of the synaptic SNARE complex consists of an extended four-helix bundle structure that contains one helix of Synaptobrevin 2 (R-SNARE), one helix of Syntaxin 1a (Qa-SNARE), and two helices of SNAP-25 (Qb- and Qc-SNAREs) [31]. The two SNAP-25 helices, and the N-terminal three-helix bundle Habc-domain [29,30] and the SNARE-motif of Syntaxin 1a are connected by flexible linker regions [18,46]. Syntaxin 1a and Synaptobrevin each contain a C-terminal TM domain, whereas SNAP-25 is attached to the membrane by palmitoyl modifications. (B) The core of the endosomal SNARE complex consists of a very similar four-helix bundle structure. It is composed of one helix each of Endobrevin (R-SNARE), of Syntaxin 7 (Qa-SNARE), of Vti1b (Qb-SNARE), and of Syntaxin 8 (Qc-SNARE) [36]. Syntaxin 7, Vti1b, and Syntaxin 8 carry large N-terminal domains, probably all constituting three-helix bundle structures [37]. All four endosomal SNAREs hold a TM domain adjacent to their respective SNARE motif [22]. The cylinders represent the TM regions that are linked by a short sequence of unknown structure to the respective SNARE helices. The curved lines represent extended flexible linker regions.

somal complex is composed of four different SNAREs, Syntaxin 7 (Qa), Vti1b (Qb), Syntaxin 8 (Qc), and Endobrevin (R), which all possess a TM region adjacent to their respective SNARE motif [22]. In addition, not only Syntaxin 7, the homolog of Syntaxin 1a, but alsoVti1b and probably Syntaxin 8 carry N-terminal three-helix bundle domains [37] (Fig. 1B).

3. Molecular steps of SNARE assembly

In the last years, the molecular steps of SNARE assembly (Fig. 2) have been mainly deciphered through meticulous studies of the soluble SNARE regions. These investigations have revealed the kinetically and thermodynamically favorable pathways, which most certainly are used in vivo as well.

3.1. A conformational change of Syntaxins precedes SNARE assembly

Syntaxin 1a can form a tight 1:1 complex with Munc18 (also referred to as nSec1) [38]. The crystal structure of this complex has revealed that the N-terminal half of the Syntaxin SNARE motif packs against a groove of the Habc-triple-helix bundle, forming a so-called "closed conformation" of Syntaxin1a [39].

A similar closed conformation was discovered for the yeast Syntaxin-homolog Sso1p. Sso1/2p is involved in the fusion of Golgi-derived vesicles with the plasma membrane, where it forms a SNARE complex with the SNAP-25-homolog Sec9p and the Synaptobrevin-homolog Snc1/2p ("yeast exocytotic" SNARE complex). Removal of the Habc-domain of Sso1p was shown to accelerate SNARE assembly about 2000-fold [20]. This implies a tight interaction of the Habc-domain of Sso1p with its own SNARE motif, slowing down the interaction with the SNARE partners. Indeed, the two domains of Sso1p purified as individual fragments can interact [21]. Finally, the crystal structure of Sso1p confirmed that it exhibits a closed conformation similar to the one of Syntaxin 1a in complex with Munc18 [40]. Since a nuclear magnetic resonance (NMR) study indicated that Syntaxin 1a in isolation exhibits a closed conformation as well [41], it was expected that all Syntaxins primarily exist in a closed conformation. However, several Syntaxins were found to exhibit an open conformation [42–44]. This raises the question whether the closed conformation is only a special adaptation of some Syntaxins. Reexamination of neuronal Syntaxin 1a then indicated that the closed conformation may not be predominant since removal of its Habc-domain accelerates the interaction with SNAP-25 only about 10-fold [45]. In fact, single molecule fluorescence resonance energy transfer (FRET) studies confirmed that most Syntaxin 1a molecules are in the open conformation (about 70%) [45]. Thus, the role of the conformational change of Syntaxin 1a needs to be reevaluated.

Structural comparison of Sso1p with the synaptic SNARE complex indicates that the closed conformation is mutually exclusive with binding to Sec9p [40]. Consequently, Syntaxins must be open before SNARE assembly [20]. Characterization of the synaptic and the yeast exocytotic SNARE complexes by thermal denaturation experiments revealed that the Habc-domain is not in tight contact with the central SNARE bundle [15,19,20]. In addition, the linker

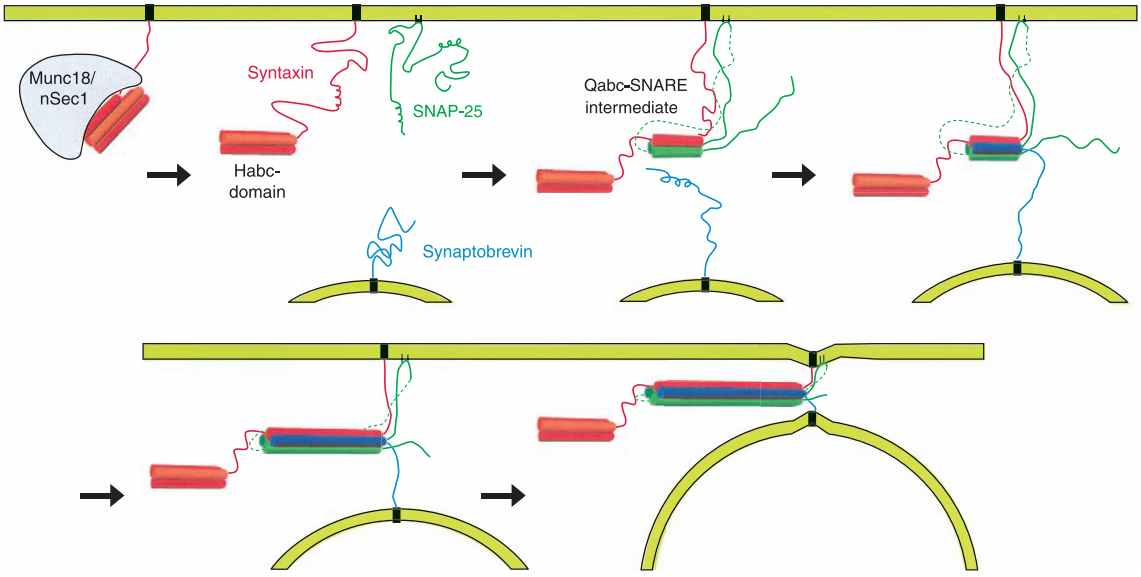

Fig. 2. A refined model of SNARE protein mediated membrane juxtaposition. The assembly of the SNARE proteins involved in synaptic exocytosis is best characterized. Syntaxin 1a and SNAP-25 reside in the plasma membrane, but presumably not as preformed complex. Munc18 can keep Syntaxin in its closed conformation and thereby controls SNARE-assembly because Syntaxin can bind only in its open conformation to SNAP-25. Before assembly, the SNARE motifs of the three SNARE proteins are flexible and mostly unstructured. In a rate-limiting nucleation step the three Q-SNARE helices of Syntaxin and SNAP-25 start to interact at their respective N-termini. Subsequently, the R-SNARE Synaptobrevin from the synaptic vesicle binds into the Qabc-SNARE-intermediate and final zippering occurs. Upon assembly the synaptic vesicle is pulled towards the plasma membrane. Whether SNARE assembly proceeds into the TM regions, thus mediating membrane fusion without the aid of additional factors is so far unclear.

region between the Habc-domain and the SNARE motif of Syntaxin 1a was shown by electron paramagnetic resonance (EPR) spectroscopy to be highly flexible when it is in the SNARE complex [46]. Taken together, this indicates that no strong interaction takes place between the Habc-domain and the core SNARE complex. The flexible linker between Habc-domain and the core complex might help to bring up other controlling factors that bind to the Habc-domain, for example Munc13, to the site of assembly.

3.2. An intermediate is required on the assembly pathway

During assembly of the yeast exocytotic SNARE complex, Sso1/2p must interact with Sec9p before Snc1/2p can bind [20]. Yet, it seemed possible that the observed SNARE assembly pathway is only dictated by the preceding conformational change of the Syntaxin homologue. A similar pathway was found, however, when only the SNARE motifs of the synaptic SNAREs were investigated [26]. Denaturation experiments showed that the synaptic SNARE complex does not refold at conditions where unfolding occurs. Interestingly, its folding requirements are similar to the ones of the Syntaxin/SNAP-25 complex. The view that SNARE assembly requires the formation of a Syntaxin/SNAP-25 intermediate was corroborated by a kinetic analysis of the refolding process [26]. This demonstrates that the pathway of assembly does not depend on the open/closed conformational change of Syntaxin, but is encoded in the SNARE motifs.

Sso1/2p and Sec9p form a 1:1 complex, which was proposed to constitute a three-helix bundle structure that serves as a binding site for Snc1/2p [20,21]. NMR data showed that the helical structure of Sso1p in complex with Sec9p extends from the N terminus until about two thirds of the SNARE motif, the remaining C-terminal portion being unstructured [21]. It is therefore likely that the C-terminal regions of both Sec9p-SNARE motifs in the complex are unstructured as well.

The analogous Syntaxin/SNAP-25 complex consists of two molecules of Syntaxin 1a and of one molecule SNAP-25 [15]. The overall structure of this complex is a four-helix bundle similar to the one of the synaptic SNARE complex, but with a second Syntaxin molecule occupying the binding site of Syntaptobrevin [18,47,48]. Moreover, in contrast to the Ssop/Sec9p complex, the Syntaxin/SNAP-25 complex is only slightly unstructured at both ends of the helix bundle. Thus, it looks as if the formation of both SNARE complexes requires an analogous intermediate, but with a different composition and structure. However, the fact that in the Syntaxin/SNAP-25 complex the binding site for Synaptobrevin is held by a second Syntaxin molecule raises the possibility that this structure may be off-pathway; intuitively, it seems awkward that Synaptobrevin would have to replace the second Syntaxin. It is likely that the presence of a second Syntaxin in the Syntaxin/SNAP-25 complex is due to the strong tendency of Syntaxin 1a to undergo less specific coiled-coil interactions. For example, the SNARE motif of Syntaxin 1a can form homo-oligomeric helix bundles [18,49]. It is also capable of forming a four-helix bundle with only the first half of SNAP-25 [50]. Furthermore, complexes of the SNARE motif of Syntaxin, Synap-

tobrevin and either the first or second half of SNAP-25 have been reported [27,28]. These complexes seem to consist of a four-helix bundle, in which a second Syntaxin molecule has occupied the binding site of the respective absent SNAP-25 helix (Dirk Fasshauer, unpublished observations). So far, none of these complexes has been shown to be functional, suggesting that generally four different coils are required for functional SNARE-interactions. Therefore, a Syntaxin/SNAP-25 complex with a 1:1 stoichiometry, i.e. a "Qabc–SNARE complex", may resemble more closely the functional on-pathway intermediate. Indeed, a detailed kinetic analysis of SNARE assembly indicates that a transient 1:1 interaction of Syntaxin 1a and SNAP-25 serves as true binding site for Synaptobrevin [51].

The endosomal SNARE complex exhibits a hysteresis similar to the one of the synaptic complex [26], indicating that its assembly requires an intermediate as well. But it is unclear whether the endosomal folding intermediate is equivalent to the Qabc–SNARE intermediates of exocytotic SNAREs. In the endosomal SNARE complex the two helices of SNAP-25 are contributed by two independent proteins, Vti1b and Syntaxin 8 [22,36], which would allow for folding intermediates of different compositions. It is therefore important to clarify whether all SNARE complexes use a similar assembly pathway. In addition, it would be interesting to know whether folding intermediates observed for soluble proteins act indeed, like Syntaxin and SNAP-25, as one unit when on one membrane. Analysis of the composition of SNAREs on the target and vesicular membrane cast doubt on such a universal SNARE assembly pathway (Ref. [52] and references therein), but more detailed investigations are necessary.

3.3. N-terminal zippering

For topological reasons, it seems natural to assume that SNARE assembly between membranes starts from the N termini of the proteins [11,12]. Nevertheless, until recently, only circumstantial evidence was available for such a "zipper" mechanism. Since only the N-terminal portion of the Ssop/Sec9p complex is structured, it was suggested that binding of the R-SNARE Snc1/2p might start N-terminally [21]. This was also deduced from Deuterium exchange experiments indicating that the N-terminal portion of the synaptic SNARE complex is more tightly packed [53]. In addition, threonine and serine side chains, which are rare in other leucine zippers, are frequently incorporated into hydrophobic layers of SNARE complexes, where they form intramolecular hydrogen bonds. Formation of these bonds may assist in a directed assembly process [36]. An N-terminal start of the assembly was also inferred from the observation that a monoclonal antibody against the N-terminal portion of SNAP-25 inhibits SNARE complex formation [54]. Further evidence that the primary pathway for SNARE assembly starts from the N termini comes from kinetic experiments in which the SNARE motifs of synaptic SNAREs were shortened [51]. C-terminal truncations did not significantly alter the assembly process, whereas assembly was inhibited by N-terminal truncations of SNAP-25 or of the SNARE motif of Syntaxin. Furthermore, assembly required Syntaxin and both SNAP-25 helices. Apparently, all three Q-SNARE-helices are strictly necessary for formation of the folding nucleus, indicating that both SNAP-25-helices cooperate during assembly. This might explain the joining of two SNARE motifs in SNAP-25. No difference, however, was observed for N- or C-terminally truncated Synaptobrevin [51], substantiating that nucleation is independent of the R-SNARE. In vitro, formation of the synaptic Qabc-intermediate appears to be transient since the open binding site can immediately be occupied by a second Syntaxin helix. In vivo, Synaptobrevin could thus either bind directly upon formation of the Qabc–SNARE intermediate or this intermediate may have to be stabilized by other proteins.

Remarkably, there are conspicuous parallels between the formation of SNARE complexes and the well-studied assembly of the GCN4 leucine zipper, a dimeric coiled coil. Because the formation of the leucine zipper is accompanied by a transition of unfolded monomers into a two-helix bundle, it is studied as a simple model for coupled folding and association. Although the exact order of molecular events during association is still under debate, it appears that small "trigger sequences", which adopt a helical conformation, exist within the coiled-coil domains [55]. Leucine zipper formation arises then from the establishment of a confined α-helical transition complex, from which zippering proceeds into the helix bundle (Refs. [56,57] and references therein). Correspondingly, it is likely that the trigger sequence of SNARE assembly is predominantly confined to the N-terminal tips of the Q-SNARE helices. It needs to be evaluated in the future at which point the R-SNARE interacts with this Qabc–SNARE intermediate, leading to a quasi-irreversible interaction [26].

In solution, SNAREs assemble with the relatively slow rate of about 6000 $M^{-1}s^{-1}$ [20,45,51]; between liposomes, comparable rates were observed [13]. This slow rate can be explained by the complicated nucleation step and, presumably, the time required for the subsequent zipper process can be neglected.

3.4. Partially assembled SNARE complexes

To merge two lipid bilayers, SNARE assembly must overcome an energy barrier of yet unknown height [58]. The presence of a marked hysteresis between folding and unfolding prevented the direct measurement of the free energy of SNARE assembly [26]. Hence, the energetic contribution of SNARE assembly towards membrane fusion remains to be clarified. Possibly, SNARE assembly primarily catalyzes the apposition of the membranes. A scenario can be imagined in which the SNARE assembly and the repulsive forces between membranes balance each other and

assembly comes to a halt. One cannot predict, however, whether partially assembled *trans*-SNARE complexes occur as stable intermediates before final fusion is triggered. Likewise, it is impossible to deduce to which layer of the SNARE bundle assembly would proceed—even though the hydrophilic "0-layer" in the center of the helix bundle is an attractive candidate.

Partially assembled *trans*-SNARE complexes may be targets for modifying factors that would prevent or trigger further zippering. For example, it has been suggested that the soluble protein Complexin interacts primarily with *trans*-SNARE complexes, which may be stabilized thereby [53,59,60]. Furthermore, it is conceivable that the action of clostridial neurotoxins, a family of highly specific SNARE proteases, can largely be explained by an attack of trans complexes, in which the C-terminal cleavage sites are still unprotected.

The tightest packing of the SNARE bundle is observed in the N-terminal portion [53] Consequently, stable complexes can be assembled with C-terminally truncated SNAREs [18,53]. Taken together, these data indicate that, in principle, partially assembled complexes could exist, though their existence in vivo remains to be demonstrated (for a more detailed discussion see Refs. [7,10]).

4. In vitro fusion systems

In vitro, SNARE-mediated membrane fusion has been characterized either using (semi-) intact cellular compartments [5,61] or artificial liposomes containing purified SNARE proteins [13]. Since cellular compartments are very heterogeneous in their composition, fusion activity can only be indirectly related to SNAREs. In contrast, artificial proteoliposomes are better defined and thus should faithfully report the activity of SNARE proteins. Yet, since this fusion assay is based on fluorescence quenching, fusion of the liposomes is observed and not the proteins believed to mediate the process. Although these experiments suggest that SNAREs constitute a minimal membrane fusion machinery, it is necessary to characterize SNAREs in membranes in greater detail in order to gain mechanistic insights.

4.1. Structure of the linker region between SNARE motif and TM domain

The short linker region connecting the core complex and the TM region is likely to play an essential role during the yet unknown mechanism by which the force of SNARE assembly is harnessed for membrane fusion. Whether the linker region is stiff or flexible would fundamentally change the role of SNAREs during membrane fusion: Through a stiff extension of core complex helices into the TM helices, the force of SNARE assembly might directly lead to membrane fusion. A flexible linker would be intuitively more difficult to reconcile with an active fusion mechanism.

To decide what kind of fusion mechanism is employed, Rothman and colleagues extended the linker between the TM region and the coiled coil region of SNAREs and tested their ability to fuse liposomes [62]. An approximately 50%-reduced fusion activity was observed when five additional flexible residues (>15 Å in an extended conformation) were introduced into Syntaxin. Additional extension of the linker further reduced the fusion activity. Similar experiments were carried out with Synaptobrevin, but strangely the fusion activity appeared to be much less perturbed [62]. In a yeast vacuolar fusion assay, similar experiments have been carried out on the Syntaxin homolog Vam3p. Introduction of only three residues between SNARE motif and TM domain of Vam3p reduced vacuolar fusion to about 60%. Longer linkers gradually reduced fusion efficiency [63]. Together, these data may suggest that fusion occurs without an active role of the TM regions while the membranes are held into close apposition by the SNARE bundle. Nevertheless, replacement of the TM domains of the synaptic SNAREs by covalently attached lipid anchors completely inhibited fusion in the liposome fusion assay [64]. Similarly, expression of Sso2p or Snc1p, in which the TM domain was substituted with a geranylgeranyl modification, abolished secretion in yeast [65]. This implies a more active role of the TM region during fusion, possibly a direct conformational coupling between TM domain and core complex [64]. However, it cannot be excluded that the TM domains only rendered the membranes more fragile and thus prone to fusion while they are kept in close apposition. Therefore, the question how the release of energy upon SNARE assembly is used for lipid bilayer fusion remains unanswered.

Recently, a stretch of only 12 residues in the linker region and the N-terminal half of the TM segment of Syntaxin 1a were investigated by EPR spectroscopy [66,67]. The measurements performed suggest that the basic residues of the linker region of Syntaxin are unstructured and penetrate into the membrane–water interface. A stronger helical tendency of this region, however, was observed by molecular dynamics simulations [68]. Interestingly, the presence of charged lipids, hence a more natural membrane environment, was found to strongly increase the helical tendency of the linker region [68], which so would allow for substantial mechanical coupling.

When the Syntaxin/SNAP-25 complex was investigated by EPR spectroscopy, no change was found in the structure of the linker region in comparison to individual Syntaxin. Interestingly, the Syntaxin/SNAP-25 complex in the membrane contains, like the soluble complex, two Syntaxin molecules [67]. To "mimic" a *trans*-SNARE complex, Syntaxin was studied in complex with SNAP-25 and a Synaptobrevin that did not contain a TM domain. Again, the structure of the linker region was not altered upon complex formation [66]. Unfortunately, no measurements were carried out in the presence of the TM region of Synaptobrevin. Therefore, it is unclear whether structuring of the linker region(s) may occur upon complete zippering

of the SNARE bundle into the TM regions. In addition, it remains undetermined to what extent the oligomerization of Syntaxin [66]—note that the presence of the TM domain substantially intensifies this tendency [69]—may have influenced the EPR measurements.

Nevertheless, penetration of basic residues into the membrane–water interface is common for many single-spanning membrane proteins [70]. Thus, in this respect, the SNARE protein Syntaxin appears to be an ''ordinary'' membrane protein without any obvious structural specialization of a fusion factor. Interestingly, studies of synthetic peptides of both Syntaxin and Synaptobrevin TM segments inferred structural flexibility of these regions [71]. Thus, it is possible that a flexible rather than a stiff linker between four-helix bundle and TM regions can contribute as well to the observed fusion activity of SNAREs. However, a more comprehensive analysis of the TM region, the adjacent region, and the cytosolic portion of Syntaxin and Synaptobrevin is required.

4.2. Specificity of SNARE assembly

In vitro, it is possible to assemble stable non-cognate SNARE complexes, i.e. complexes that do not occur in vivo, by exchanging one helix from one SNARE subfamily by another helix from the same subfamily [72,73]. This is not surprising given the high conservation of the interacting layer residues in the interior of the bundle [31,33,36]. Thus, functional SNARE complexes can form when four different SNARE helices from each of the four different subfamilies are present (''RQabc–SNARE complex''). Accordingly, SNARE interaction is less specific than originally proposed [1,14]. The SNARE machinery is at the end of a series of protein–protein interactions that preserve the specificity of a given membrane fusion step. It is thought that SNAREs have evolved to transfer the energy of assembly into the mechanical strength required to appose/merge lipid bilayers. In fact, little selectivity can be expected for such a machinery. Since SNARE assembly likely occurs unidirectionally [26], the choice of SNAREs must essentially occur prior to assembly. Therefore, it is unlikely that SNAREs participate in a ''proofreading'' process during assembly. It is not surprising, however, that SNAREs are much more selective in their natural environment [74], where upstream operating proteins mediate the specificity of vesicular trafficking.

Remarkably, Rothman and colleagues have proposed that SNARE interactions are highly specific, based on the liposome fusion assay [75,76]. The discrepancy between experiments carried out with only the soluble SNARE-domains [72,73] or with SNAREs incorporated into liposomes [75,76] can be puzzling. A closer inspection of the SNARE combinations analyzed in the liposome fusion assay reveals that more than 65% of the actually tested SNARE sets did not obey the aforementioned rule that four different SNARE coils are strictly required to function as a SNARE complex [75,76]. The totality of these sets, however, was used to account for SNARE specificity. This is not clear since the old nomenclature of v- and t-SNAREs was applied, which can be misleading [33].

In about 35% of the experiments ''correct'' SNARE combinations were tested. About half of these combinations were able to fuse liposomes—even if SNAREs were mixed that probably do not interact in vivo. Thus, in total less than 20% of the tested combinations appear to be truly specific.

One explanation for this occasionally observed SNARE specificity might be that the lipid bilayer induces a change in the structure of the SNARE motifs, rendering them incapable of forming a non-cognate SNARE complex [75]. Yet it is largely unknown how the overall structure of SNARE proteins is influenced by the lipid bilayer. It should be stressed that the known properties of soluble SNARE domains are unlikely to be dramatically altered in membranes: Although the lipid bilayer may modify the structure of the region adjacent to the TM domain, it is rather implausible that the properties of the distant cytosolic portion of a SNARE protein will be drastically changed—if this portion does not interact directly with the membrane or its own membrane anchor.

Another more likely explanation for the partially observed true SNARE specificity might lie in the inability of the proteins to assemble via a given pathway. For example, the endosomal Syntaxin 7 does not form a stable complex with the neuronal SNAP-25 (Wolfram Antonin, unpublished observation). Hence, SNARE sets, which were found to be incapable to mediate liposome fusion but were ''structurally correct'' [75,76], might possibly also not assemble in solution.

Taken together, in view of these considerations, it appears to be somewhat far-fetched to claim that the ''compartmental specificity of cellular membrane fusion [is] encoded in SNARE proteins'' [75]. Of course the SNARE machinery is specific, since it requires four different SNARE motifs to form a functional four-helix bundle, but exchange of helices is apparently widely tolerated. This is best exemplified by the fact that in vivo at least some SNAREs can participate in different SNARE interactions [77–80].

4.3. Proposed stages of SNARE assembly between liposome membranes

Removal of the N-terminal Habc-domain of Syntaxin accelerates liposome fusion [81]. In all experiments, Syntaxin and SNAP-25 were co-reconstituted into liposomes, implying that Syntaxin is already in the open conformation. To explain the observed change in the fusion rate, Rothman and colleagues proposed that the Habc-domain exerts a second inhibitory function by blocking the binding site of Synaptobrevin in the Syntaxin/SNAP-25 complex [81]. There is so far, however, no indication for such an interaction between the Habc-domain and the Syntaxin/SNAP-25 complex. On the contrary, no interference of the Habc-

domain with Synaptobrevin binding could be observed in solution (Ref. [20] and own unpublished observations). If the Habc-domain would indeed block the binding site of Synaptobrevin, one would assume that the Habc-domain in isolation should also be able to compete with Synaptobrevin and thus inhibit fusion. Unfortunately, this has not been tested, and it therefore cannot be excluded that the large Habc-domain interferes with the fusion process for steric reasons.

Furthermore, it was shown that liposome fusion is blocked by low temperature (4 °C), but is twice as fast after a prolonged 4 °C-pre-incubation. In the latter case, it was suggested that partially assembled *trans*-SNARE complexes between liposomes accumulate [81,82]. This should intuitively result in a higher number of morphologically docked liposomes, but has never been confirmed by electron microscopy. The 4 °C-block was claimed to occur only in the presence of the Habc-domain, but apparently no experiment was carried out that demonstrates the effect of low temperature on liposome fusion in the absence of this domain. Thus, it remains elusive whether the 4 °C-block is indeed, as claimed, a ''frozen'' SNARE assembly step or just a result of an altered fluidity of the membrane.

Moreover, it is puzzling why fusion after a 4 °C-preincubation is not much faster after all. Such preincubated liposomes, which are believed to be ''ready to fusion'' state—morphologically docked through partially assembled *trans*-SNARE complexes—fuse more than twice as slow (≈ 2.5 ''rounds''/2 h) than liposomes carrying the SNARE motif of Syntaxin (≈ 5.5 ''rounds''/2 h).

This difference is again explained by the elusive interaction of the Habc-domain with the Syntaxin/SNAP-25 complex: the Habc-domain would allow for the establishment of *trans*-SNARE complexes with the N-terminal portion of Synaptobrevin bound, but still for final zippering the Habc-domain would have to be displaced from this *trans*-complex.

This model was deduced from experiments in which peptides of the soluble domain of Synaptobrevin were added to the fusion assay [82]. Addition of the N-terminal half of Synaptobrevin was shown to inhibit fusion, probably because it interferes with the N-terminal start of Synaptobrevin assembly. In contrast, addition of the C-terminal half of Synaptobrevin accelerated fusion. It was suggested that the C-terminal peptide displaces the Habc-domain [82]. Yet, as outlined above, there is no indication for an interaction of the Habc-domain with the Syntaxin/SNAP-25 complex. In addition, it was suggested that binding of the C-terminal Synaptobrevin peptide is structuring the C-terminal portion of the *trans*-SNARE complex [82]. Why this should lead to faster fusion, however, is not plausible; the peptide would obstruct the pathway for fusion because it needs to be displaced by Synaptobrevin during the final zippering process.

Interestingly, investigations on the Syntaxin/SNAP-25 complex have established that it accommodates two Syntaxin molecules [15,18,47,48,67]. This fact may be the key for an alternative interpretation of several findings. Both Synaptobrevin peptides can bind to the Syntaxin/SNAP-25 complex; upon binding they would displace the second Syntaxin molecule, which occupies the binding site of Synaptobrevin. Synaptobrevin-loaded liposomes would then encounter two different situations: The N-terminal Synaptobrevin-peptide would block the site to which Synaptobrevin would start to assemble into the Syntaxin/SNAP-25 complex, thus slowing down fusion. In contrast, the C-terminal peptide would bind to the C-terminal portion of the Syntaxin/SNAP-25 complex and free the ''N-terminal assembly site'', which is normally occupied by the second Syntaxin helix. To this complex, full-length Synaptobrevin could more easily bind and, upon further assembly, would probably displace the C-terminal peptide.

Taken together, it seems therefore that the experimental foundation for a second inhibitory role of the Habc-domain of Syntaxin is unsatisfactory, although such a yet unknown role would be fascinating. These uncertainties should be addressed in future studies because they hamper a clear analysis of SNARE assembly between membranes. Furthermore, one has to keep in mind that several of the findings of the liposome fusion assay are based on sometimes very subtle kinetic differences. Kinetic measurements build on the exact determination of the protein concentration and the uniform distribution of the proteins. This seems to be rather difficult to achieve for proteoliposomes since proteins can have a wrong orientation in the membrane or can form aggregates in which they may be incapable to form a SNARE complex. Therefore, in summary, the analysis of the SNARE assembly steps appears to suffer greatly from an insufficient characterization of the initial status of SNAREs in liposome membranes.

5. Conclusions

According to the ''zipper'' model, sequential formation of SNARE complexes between vesicular and target membranes pulls the lipid bilayers into close proximity, ultimately promoting fusion [11,12]. Using only the basic constituents—SNAREs and membranes—it has been difficult, however, to decide whether SNAREs are indeed fusion factors or whether they only bring the membranes into apposition. In fact, shortly after the proposal of the zipper model, it had been shown that SNARE interaction between membranes suffices for bilayer merger [13], unfortunately by using an assay designed to monitor liposome fusion rather than the fusion machinery. Since, in addition, the structure and the oligomeric status of the SNARE proteins incorporated into liposome membranes have been insufficiently characterized, the conclusions drawn from this assay remain ambiguous.

In solution, the SNAREs involved in synaptic exocytosis have been extensively studied and thus serve as paradigm

for other SNAREs. Their assembly is accompanied by a transition from unstructured monomers into a highly stable four-helix bundle structure. It is likely that the energy released during this process is used for membrane apposition/merger. Formation of the synaptic SNARE complex is highly orchestrated. It is initiated by the interaction of the N-terminal portions of three Q-SNAREs helices which reside in the plasma membrane. This Qabc-SNARE intermediate probably provides the high affinity binding site for the R-SNARE Synaptobrevin in the synaptic vesicle membrane. Thus, indeed, SNARE assembly begins with the interaction of the membrane-distal regions of the SNARE proteins.

The establishment of this Qabc-intermediate is a kinetic bottleneck during SNARE assembly. It is probable that, after first contact, the proteins zipper up quickly into the final four-helix bundle structure. Therefore, current assays that measure SNARE assembly in solution or between liposomes very likely solely observe the rate of intermediate formation. Any extrapolation regarding the faster molecular steps that follow the initial contact—zippering and fusion—has thus to be taken with considerable caution.

Interestingly, Syntaxin 1a and SNAP-25 appear in vivo to be present in different areas of the plasma membrane, suggesting that their majority do not exist as preformed complexes [83,84] that await the binding of Synaptobrevin. Yet, they are capable of forming SNARE complexes when exogenous Synaptobrevin is added [85]. It seems thus conceivable that in vivo as well as in vitro SNARE assembly is highly orchestrated. One likely control mechanism for SNARE assembly would be the conformational change of Syntaxin, which regulates the accessibility of its own SNARE motif.

In conclusion, the structural and biophysical properties of SNARE proteins indeed led them to appear perfectly suited to catalyze the juxtaposition of two membranes in a zipper-like fashion. Nevertheless, mainly because SNARE proteins in membranes have only been characterized partially, it remains speculative whether SNARE assembly is sufficient to drive membrane merger.

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